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Intracellular Neutralization of Shiga Toxin 2 by an A Subunit-Specific Human Monoclonal Antibody▽†

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Infection of children with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is the leading cause of hemolytic-uremic syndrome (HUS). Stx2, one of two toxins liberated by the bacteria, is directly linked with HUS. We have previously shown that Stx2-specific human monoclonal antibodies (HuMAbs) protect mice and piglets from fatal systemic complications of Stx2. The present study investigates the mechanisms by which our most efficacious A- and B-subunit-specific HuMAbs neutralize the cytotoxic effects of Stx2 in vitro. Whereas the B-subunit-specific HuMAb 5H8 blocked binding of Stx2 to its receptor on the cell surface, the A-subunit-specific HuMAb 5C12 did not interfere with the toxin-receptor binding. Further investigations revealed that 5C12 did not block endocytosis of Stx2 by HeLa cells as both Stx2 and 5C12 colocalized with early endosomes. However, 5C12 blocked the retrograde transport of the toxin into the Golgi and the endoplasmic reticulum, preventing the toxin from entering the cytosol where the toxin exerts its cytotoxic effect. The endocytosed 5C12/Stx2 complexes appear to be rapidly transported to the plasma membrane and/or to the slow recycling perinuclear compartments, followed by their slow recycling to the plasma membrane, and release into the extracellular environment.

Infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) can become life threatening if it induces systemic complications, mainly hemolytic-uremic syndrome (HUS), the leading cause of acute renal failure in children (2, 11, 21, 25). Of Stx1 and Stx2, the two immunologically distinct Stxs produced by STEC, strains producing only Stx2 are more frequently associated with HUS (10, 27). Stx1 and Stx2 are similar in basic structure, binding specificity, and mode of action (9). The Stx molecule consists of an A-subunit monomer and a B-subunit pentamer. The pentameric B subunit binds to its cell surface receptor CD77, also called globotriaosylceramide (Gb_3). This triggers endocytosis of the holotoxin, mainly through clathrin-coated pits (16). Internalized Stx is then delivered to the trans-Golgi network, where it is carried by retrograde transport to the endoplasmic reticulum (ER), and then to the cytosol (28). During this process, the A subunit is nicked by the membrane bound protease furin, generating a catalytically active N-terminal A1 fragment, while a C-terminal A2 fragment remains linked by a disulfide bond (28). This disulfide bond is subsequently reduced to release the active A1 component. The released A1 fragment has RNA *N*-glycosidase activity that removes a single adenine from the 28S rRNA, thereby inhibiting protein synthesis in the intoxicated cells (7).

Several therapeutic approaches that attempted to neutralize Stx either in the gut or in the circulation include the use of synthetic Gb_3 analogues, genetically manipulated bacteria expressing Gb_3, and Stx-specific neutralizing antibodies (32). The systemic administration of Stx-specific neutralizing antibodies, we believe, is currently the most promising approach for prevention of Stx-mediated systemic complications including HUS.

The production, characterization, and evaluation of a panel of human monoclonal antibodies (HuMAbs) against Stx1 and Stx2 in transgenic mice was shown to effectively inhibit cytotoxicity in HeLa cells and protect mice and piglets (22, 23). Stx2 A-subunit-specific HuMAb, 5C12, currently undergoing phase I clinical trials, was selected on the basis of its superior efficacy in protecting mice against lethal challenge with Stx2 (30) and Stx2 (31) variants. In orally infected piglets with STEC, as in children, diarrheal symptoms precede systemic complications associated with Stx2 uptake from the gut. The HuMAb 5C12 protects piglets against fatal CNS symptoms when administered well after onset of diarrhea following bacterial challenge (48 h postchallenge) (30). Furthermore, 5C12 protects 20 to 40% mice even when administered 1 to 2 h after intravenous challenge with a lethal dose of Stx2 but the B-subunit-specific HuMAb 5H8 does not protect any mice (unpublished data). In contrast, both 5C12 and 5H8 provide complete protection when administered prior to the administration of the toxin (30). These results suggest that 5C12 and 5H8 utilize distinct mechanisms of protection. In the present study, we uncover the mechanisms by which 5C12 and 5H8 neutralize the cytotoxic effects of Stx2 in vitro. We demonstrate that 5C12 but not 5H8 neutralizes toxin intracellularly, a unique mechanism of toxin neutralization.

MATERIALS AND METHODS

Cell line. HeLa cells (American Type Culture Collection, Manassas, VA) in McCoy's medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal

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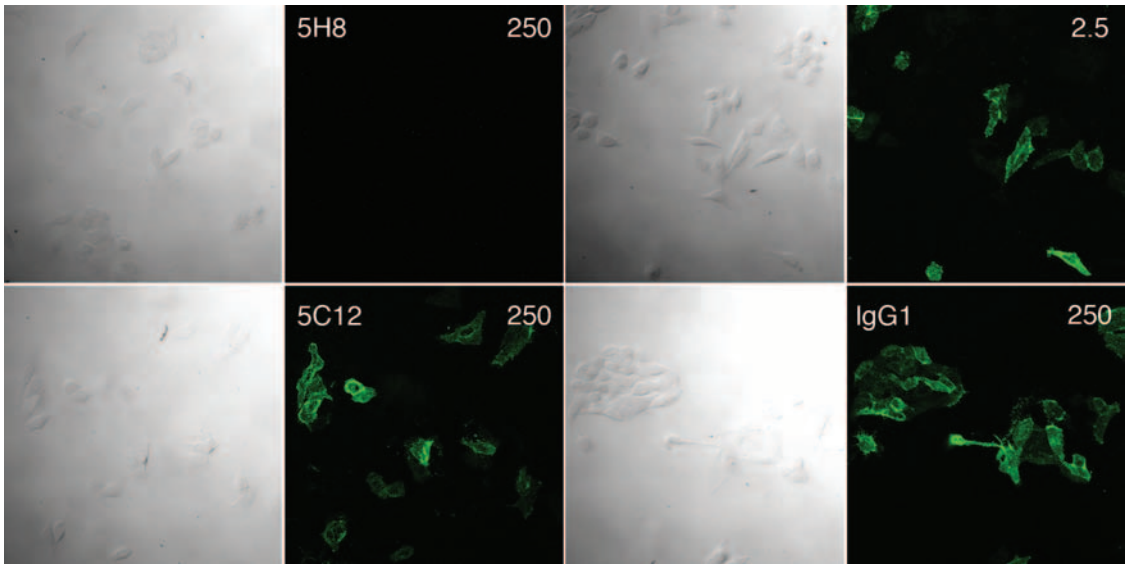

FIG. 1. Confocal microscopic analysis shows that Stx2 B-subunit-specific but not A-subunit-specific HuMAb inhibits toxin binding to the cell surface. The B-subunit-specific HuMAb 5H8, when preincubated with Stx2-AF (2.5 μg/ml), inhibited the binding of the toxin to HeLa cells at 250 μg/ml but not at 2.5 μg/ml (upper panels). The A-subunit-specific HuMAb 5C12, similar to the isotype control, did not block binding of Stx2-AF to the cell surface, even at the highest antibody concentration of 250 μg/ml (lower panels). A differential interference contrast image of each fluorescent image is shown to the left of each panel. This experiment was repeated more than three times with similar results.

bovine serum (complete medium) were grown and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Stx2. Stx2 was purified from cell culture supernatant of *E. coli* C600W containing the 933W phage as described elsewhere (5) and labeled with the green fluorescent dye Alexa Fluor 488 (AF488) using an AF488 labeling kit (Molecular Probes, Inc., Eugene, OR).

Stx2-specific HuMAbs. The production and characterization of Stx2-specific HuMAbs was previously described (22), and from those the most effective HuMAbs, 5C12 and 5H8, specific for A and B subunits, respectively, were selected (30). Since both are immunoglobulin G1κ (IgG1κ) isotype, the human myeloma IgG1κ was included as a control (Sigma-Aldrich, St. Louis, MO).

Human cellular organelle markers and secondary antibodies. Mouse MAbs specific for human cellular organelles used in the present study included anti-transferrin receptor (TfR; CD71) MAb (BD Pharmingen, San Diego, CA), a marker for sorting and recycling endosomes (RE), collectively called early endosomes (EE); MAb against CD63/lamp3/tetraspanin (BD Pharmingen), a well-established membrane component of late endosomes (LE) and lysosomes; and anti-Golgin97 MAb (Molecular Probes), a unique protein from the Golgi apparatus. Rabbit anti-protein disulfide isomerase polyclonal antibody (Stressgen, Canada) was used as a marker for the ER. Anti-human, -mouse, and -rabbit IgG conjugated with AF568 (red fluorescence) and anti-human IgG conjugated with AF488 (Molecular Probes) were used as secondary antibodies. Human Tf labeled with AF568 (Molecular Probes, CA), and rabbit anti-Rme-1 (a generous gift from Margaret Robinson, University of Cambridge, Cambridge, United Kingdom) were used to identify RE or endocytic recycling compartments, including the peripheral nuclear recycling compartment (PNRC).

Assay for Stx2 cytotoxicity. An in vitro cytotoxicity assay was used to evaluate the conditions by which the HuMAbs are able to neutralize the cytotoxic effects of Stx2. HeLa cells were cultured on 96-well plates at 10^5 cells/ml (100 μl/well) overnight at 37°C to produce ca. 75% cell confluence. Cells were then incubated with Stx2 and HuMAbs at 4°C as described in Results. After incubation at 37°C for 24 h, the percentage of cell mortality in the presence or absence of HuMAbs was assessed by crystal violet assay as described elsewhere (13). Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PF). Crystal violet solution was added to the cells, and after intensive washing with H_2O and drying, the cells were lysed with 100% ethanol. The optical density at 690 nm was measured in a microplate reader.

Flow cytometry analysis of HeLa cells treated with Stx2 and HuMAbs. To determine whether B-subunit-specific 5H8 and/or A-subunit-specific 5C12 inhibits binding of Stx2 to its receptor Gb_3 on the cell surface, HeLa cell suspensions were produced by treating the cells with 0.05% trypsin–53 mM EDTA. Trypsinization has been shown elsewhere not to affect Stx binding (29). Cells were then washed once in complete medium by centrifugation at 300 × *g* and 4°C for 10 min. AF488-labeled Stx2 (Stx2-AF) at 2.5 μg/ml was preincubated at 37°C for 1 h with HuMAbs (0.125 to 250 μg/ml), cooled to 4°C, and then added to suspensions of 10^6 cells which were also precooled to 4°C. After a 30-min incubation at 4°C, the cells were washed twice in cold Hanks balanced salt solution (HBSS; Cambrex BioScience, Walkersville, MD) and resuspended in 4% PF for 15 min at 4°C. The cells were washed and resuspended in PBS. The cell-associated fluorescence (10,000 cells per treatment) was collected by using FACScalibur and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Overlay histogram analysis was performed by using either CellQuest or WinMDI 2.8 software.

To determine whether B-subunit-specific 5H8 and/or A-subunit-specific 5C12 can bind to the cell bound Stx2 (Stx2 bound to its cell surface receptor Gb_3), HeLa cell suspensions of 10^6 cells were incubated for 30 min at 4°C with Stx2 at 100 ng/ml and washed twice with HBSS. Cells were then incubated for 30 min at 4°C with 20 μg of 5C12 or 5H8/ml, washed twice with cold HBSS, and fixed with 4% PF for 15 min at 4°C. Anti-human IgG labeled with AF488 was added for 30 min at 4°C, and cells were washed twice with HBSS and resuspended in PBS. Staining with isotype control antibody was performed in parallel. The cells were analyzed by flow cytometry as described above.

To detect excretion of recycled Stx2/5C12 complexes, cells were cultured in 12-well plates for 48 h at 37°C to produce ca. 75% confluence. Cells were then incubated with Stx2-AF for 30 min on ice, washed with complete medium, and incubated with 5C12, also for 30 min on ice. Cells were then maintained at 20°C for 1 h (this allows endocytosis of Stx2/5C12 complexes but blocks further transport), washed to remove excess 5C12, and then incubated at 37°C for 4 h. In a parallel set of experiments, 5H8 was added to the medium (250 μg/ml) right before incubation at 37°C, and it was removed only after 4 h of incubation. At 0- and 4-h time points at 37°C, cells were brought to room temperature, washed with HBSS, and treated with 0.05% trypsin–53 mM EDTA. Cell suspensions were washed with complete medium, fixed with 4% PF, and resuspended in PBS. The cells were analyzed by flow cytometry as described above.

Confocal microscopy analysis on HeLa cells treated with Stx2 and HuMAbs. HeLa cells were added to eight-well tissue culture chamber slides (Lab-Tek; Nunc, Naperville, IL) and allowed to settle and grow by overnight incubation at 37°C to produce ca. 75% confluence. Live cells were treated with Stx2 and HuMAbs at various conditions as described in Results. Cells were then washed with HBSS, fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at room temperature, and then washed with Perm/Wash solution (BD Pharmingen). To detect surface and intracellular HuMAbs, fixed

cells were incubated with anti-human IgG-AF568. To determine Stx2 localization inside the organelles, fixed cells were incubated with primary mouse or rabbit antibodies specific to organelle markers for 1 h at room temperature, washed with Perm/Wash solution, and treated with anti-mouse or anti-rabbit IgG-AF568.

Confocal laser scanning was performed on a Leica TCS-SP1 system with an upright DMRBE microscope. Simultaneous double-fluorescence images were obtained by using 488-nm and 568-nm laser lines to excite AF488 and AF568 dyes, respectively, using oil-immersion objectives. Fluorescent images were selected with appropriate double-fluorescence dichromic mirror and band-pass filters. The fluorescent images obtained using AF488 and AF568 were displayed as green and red, respectively. The images were then merged and presented as a single image. Yellow areas show regions where the two fluorescent colors overlap.

Live cell microscopy. Live cell microscopy was performed to determine the kinetics of Stx2 transport from EE to the Golgi apparatus and to investigate the effect of 5C12 on intracellular transport of Stx2. HeLa cells were seeded onto microscope cover glasses (Lab-Tek) fitted for a specially designed cell culture chamber. After overnight incubation at 37°C, cells were treated with Stx2-AF for 30 min on ice, washed, and incubated with or without 5C12, also on ice for 30 min. The incubation temperature was changed to 20°C for 1 h, and cells were then washed to remove unbound 5C12. Prewarmed medium was added just before coverglasses were transferred to the cell culture chamber, which maintains physiological pH. The cell culture chamber was placed on the stage of a Axiovert fluorescence microscope (40× objective, NA = 0.75; Zeiss, Thornwood, NY) and kept at 37°C using an air curtain (Nevtek, Burnsville, VA). A target field was selected and phase-contrast images were captured every 5 min using the Metamorph software package (Universal Imaging Corp., Downingtown, PA).

RESULTS

B-subunit- but not A-subunit-specific HuMAb inhibits binding of Stx2 to its receptor Gb_3 on the cell surface. Stx2-AF at 2.5 μg/ml was preincubated at 37°C for 1 h with HuMAbs (2.5 and 250 μg/ml) and then added to the cells, which were precooled to 4°C. The B-subunit-specific HuMAb 5H8 completely blocked Stx2-AF binding to the cells at 250 μg/ml but not at 2.5 μg/ml (Fig. 1). In contrast, preincubation of Stx2-AF with the A-subunit-specific HuMAb 5C12 did not prevent toxin from binding to the cells even at the highest dose of 250 μg/ml (Fig. 1). As expected, isotype control did not inhibit binding of Stx2-AF with the cells (Fig. 1). These observations were then confirmed by flow cytometry analysis. In the absence of HuMAbs, Stx2-AF (2.5 μg/ml) labeled 98% of the cells (Fig. 2, top). The HuMAb 5H8 again blocked the toxin-labeling of the cells in a dose-dependent manner (Fig. 2, bottom). The doses of 5H8 from 250 to 16 μg/ml completely inhibited Stx2-AF binding with the cells since the cells did not show any fluorescence. At 5H8 concentrations of 8, 4, and 2 μgs/ml, 7, 89, and 96% of the cells were labeled with Stx2-AF, respectively (Fig. 2, bottom). At a concentration of 1 μg/ml, the inhibitory effect of 5H8 on Stx2-AF binding with the cells was completely abrogated since 98% of the cells fluoresced (not shown). As expected, even at the high concentration of 125 μg/ml, 5C12 did not block any binding of the toxin with the cells since 98.97% cells were labeled with Stx2-AF (Fig. 2, middle). Furthermore, the fluorescence intensity of almost all cells was stronger when they were labeled with Stx2-AF/5C12 complex than when they were labeled with only Stx2-AF.

A-subunit- but not B-subunit-specific HuMAb binds with the cell-bound Stx2. The studies described above suggested that the A-subunit-specific 5C12, if not the B-subunit-specific 5H8, should bind to those Stx2 molecules that are already bound to the HeLa cells. To investigate this, HeLa cells were incubated at 4°C with unlabeled Stx2 (1 μg/ml), washed, and treated with HuMAbs (250 μg/ml), also at 4°C. The cells were

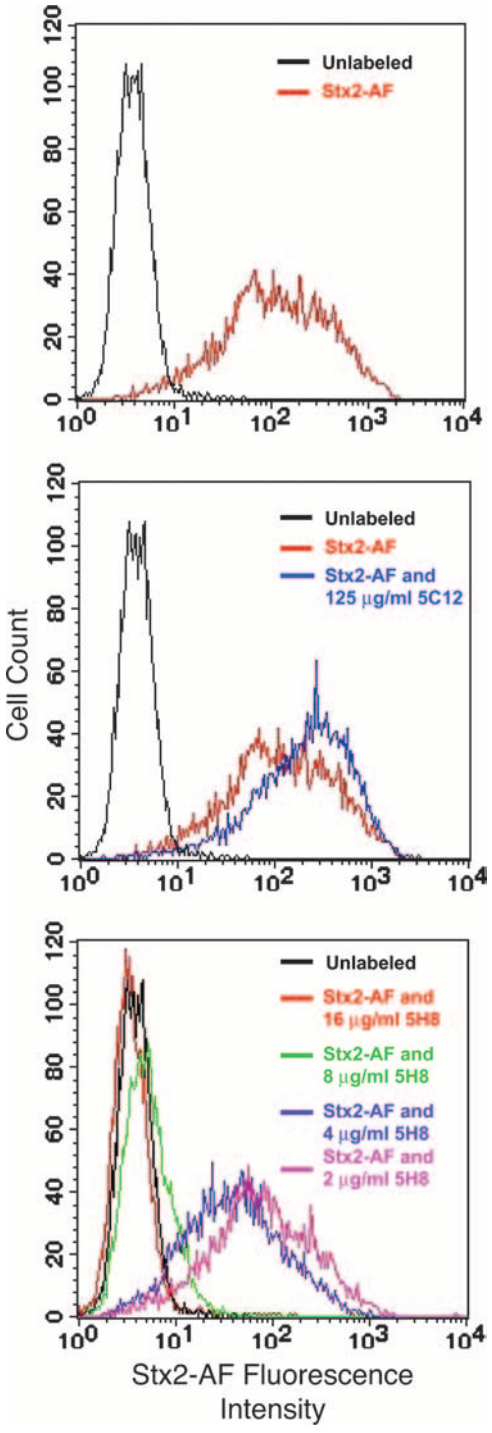

FIG. 2. Flow cytometry analysis confirms that only the B-subunit-specific 5H8 inhibits binding of Stx2 to its receptor on the cell surface. When no antibody was used, Stx2-AF labeled almost all cells (~98%) (red histogram in the top histogram overlay). Again, 5H8 inhibited cell binding of Stx2-AF in a dose-dependent manner (the bottom histogram overlay), whereas 5C12 did not prevent binding of the toxin, even at 125 μg/ml (blue histogram in the middle histogram overlay). It seems 5C12 improved the binding of Stx2 with the Gb_3 since the fluorescence intensity improved in the presence of 5C12. A total of 10,000 events were collected for each treatment. This experiment was repeated twice with similar results.

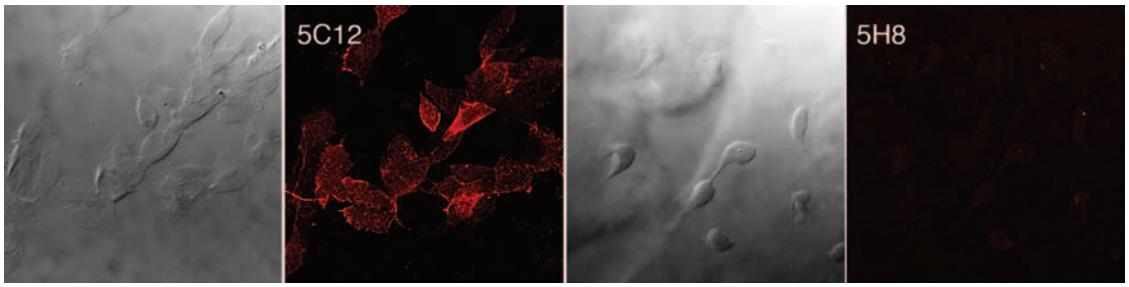

FIG. 3. Confocal microscopic analysis shows that the A-subunit-specific 5C12 but not the B-subunit-specific 5H8 binds to prebound toxin on the cell membrane. Cells which were first incubated with Stx2 and then with HuMAb showed that 5C12 (left panel), but not 5H8 (right panel), was able to bind to the cell-surface-bound Stx2, as detected by anti-human IgG-AF568. A differential interference contrast image of each fluorescent image is shown to the left of each panel. This experiment was repeated twice with similar results.

then fixed and stained with anti-human IgG-AF568. Indeed, the A-subunit-specific 5C12 but not the B-subunit-specific 5H8 bound to the cell surface-bound toxin (Fig. 3). Flow cytometry analysis also confirmed these observations since binding of 5C12 with the cell surface-bound Stx2 was detected on almost all cells (Fig. 4). Again, 5H8 and isotype control did not bind to the cell surface-bound Stx2 since the fluorescent intensity for these two antibodies was similar to the fluorescent intensity of the cells where no fluorescent marker was used (unlabeled cells, Fig. 4).

A-subunit-specific but not B-subunit-specific HuMAb protects Stx2-bound HeLa cells. We have already shown (22, 30) that the preincubation of 5C12 or 5H8 with Stx2 at 37°C for 1 h before being added to the HeLa cells protects the cells against toxin-mediated death. However, we have never shown whether 5C12 or 5H8 can protect HeLa cells if the toxin is already bound to these cells. Since our studies (Fig. 3 and 4) showed that 5C12 but not 5H8 can bind to those Stx2 molecules that are prebound to the HeLa cells, we wanted to investigate whether 5C12 could protect these cells. For this, HeLa cells were cooled to 4°C and then incubated for 30 min at the same temperature with 1.25 ng of Stx2/ml, a concentration that killed almost all cells. After excess Stx2 was removed by washing, 5H8 or 5C12 was added at various concentrations (100 to 0.8 μg/ml) and incubated for 30 min at 4°C to allow binding of the antibody with the surface-bound toxin. The cells were then shifted to 37°C, and the cytotoxicity was measured after 24 h of incubation. In agreement with the studies described above (Fig. 3 and 4), the A-subunit-specific antibody 5C12 neutralized cytotoxicity in a dose-dependent manner but the B-subunit-specific antibody 5H8 did not neutralize the toxin at any dose (Fig. 5).

5C12 binds cell-bound Stx2 at 4°C and colocalizes with it after 1 h of incubation at 37°C. To determine whether 5C12 remains with Stx2 after 1 h of incubation at 37°C when the Stx2/5C12 complex is presumably internalized, the cells were incubated with Stx2-AF for 30 min at 4°C, washed with HBSS, and incubated with HuMAb 5C12 also at 4°C for 30 min. After another cycle of washing to remove unbound Stx2-AF and 5C12, the cells were incubated at 37°C for 1 h. After washing, fixing, and permeabilizing with Cytofix/Cytoperm, cells were washed with Perm/Wash buffer and stained with anti-human IgG-AF568. The HuMAb 5C12 strongly colocalized with Stx2 (Fig. 6). Since incubation at 37°C allows internalization of Stx (12), the colocalization of Stx2 with 5C12 suggested that 5C12 was cointernalized with Stx2.

Stx2 cointernalized with 5C12 is not transported to the Golgi apparatus and instead remains in the EE. To test the

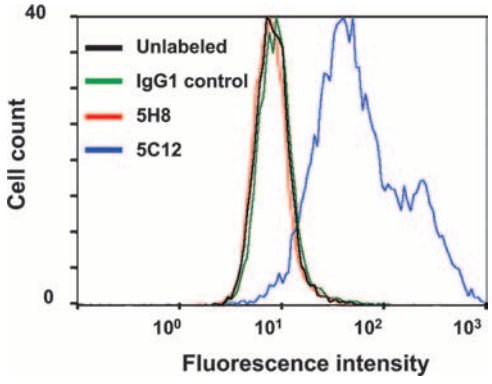

FIG. 4. Flow cytometry analysis confirms that only the A-subunit-specific 5C12 binds to the cell membrane-bound toxin. The HuMAb 5C12 (blue histogram) but not 5H8 (red histogram) bound to the cell-bound Stx2. A total of 10,000 events were collected for each treatment. This experiment was repeated twice with similar results.

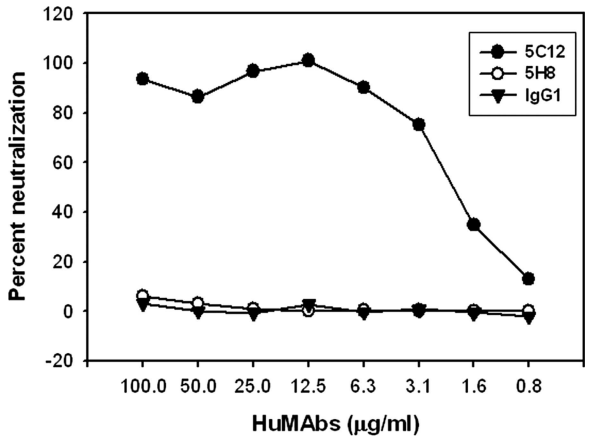

FIG. 5. The A-subunit-specific 5C12 but not the B-subunit-specific 5H8 neutralizes the cytotoxic activity of the cell-bound Stx2. The A-subunit-specific antibody 5C12 neutralized cytotoxicity in a dose-dependent manner but the B-subunit-specific antibody 5H8, similar to the isotype control (IgG1), did not neutralize the toxin at any dose. This experiment was repeated with similar results.

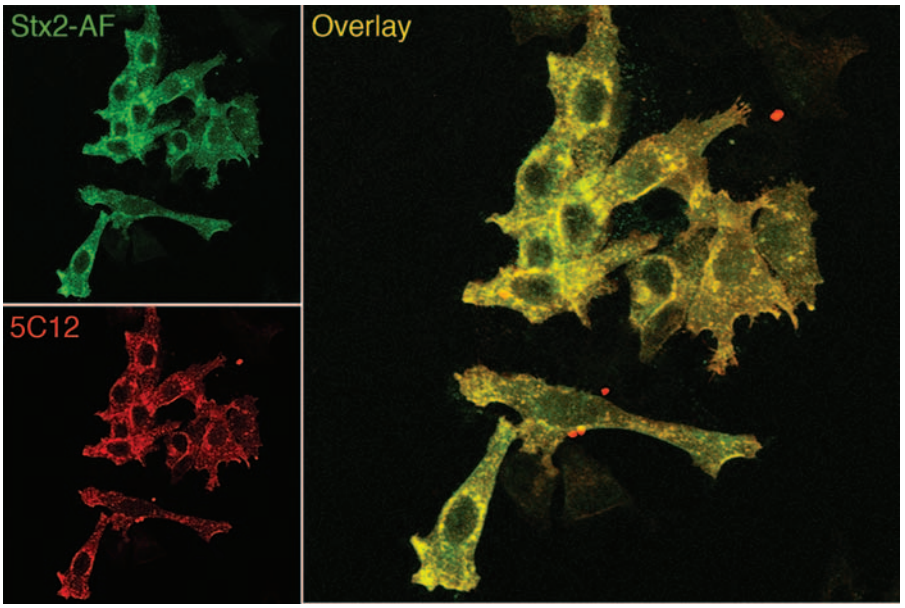

FIG. 6. The A-subunit-specific HuMAb 5C12 is cointernalized with Stx2. HeLa cells were allowed to internalize membrane-bound Stx2-AF/5C12 complexes and then analyzed by confocal microscopy. Stx2-AF is shown in green; 5C12, shown in red, was stained with anti-human IgG-AF568. The HuMAb 5C12 strongly colocalized (yellow) with Stx2 as shown in the overlay. This experiment was repeated twice with similar results.

effect of 5C12 on the retrograde transport of Stx2, we used a previously described methodology that allows peak Stx accumulation in the Golgi apparatus after 1 h of Stx incubation at 37°C with HeLa cells (8, 17). HeLa cells, precooled to 4°C, were incubated with ice-cold Stx2-AF (2.5 μg/ml) for 30 min at 4°C. Cells were washed and then incubated in the presence (250 μg/ml) or absence of 5C12 for 30 min at 4°C, and the temperature was raised to 20°C for 1 h to allow Stx2 internalization and accumulation in the EE. The temperature was then raised to 37°C for 1 h to allow Stx2 to accumulate in Golgi. Cells were fixed and/or permeabilized and then stained with anti-Golgin97 MAb (a Golgi marker) or anti-TfR (an EE marker) or anti-human IgG labeled with AF488 (to detect 5C12). As expected, cells not treated with 5C12 transported Stx2-AF to their Golgi apparatus (Fig. 7, −5C12). In contrast, in 5C12-treated cells, Stx2-AF localized to compartments other than the Golgi apparatus (Fig. 7, +5C12). These compartments were the EE since both Stx2-AF and 5C12 in the Stx2/5C12 complex colocalized with TfR after 1 h of incubation at 37°C (Fig. 8, upper and lower panels). The extension of incu-

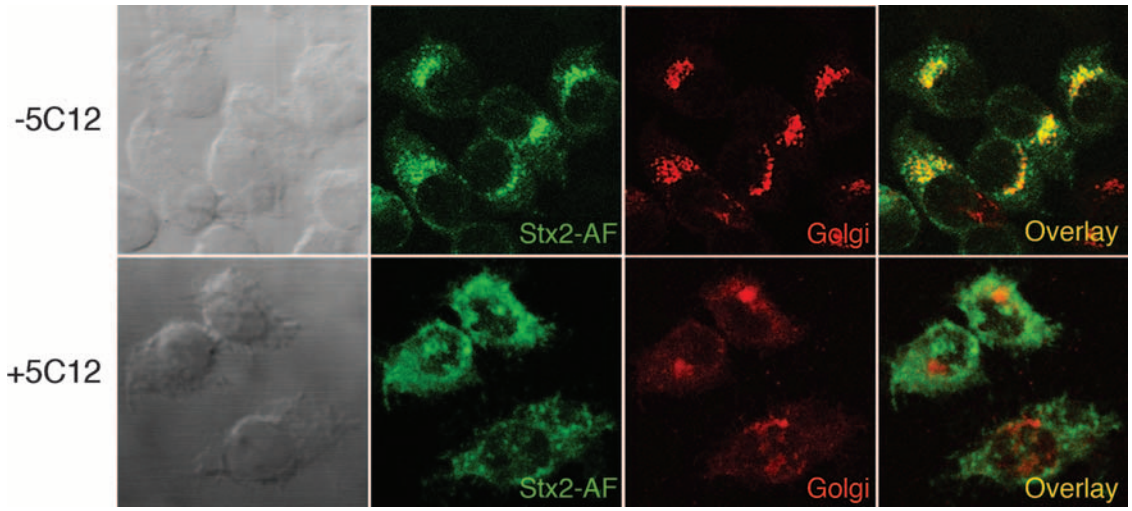

FIG. 7. Stx2 but not Stx2/5C12 complex is transported retrogradely to the Golgi compartment. In cells treated with Stx2-AF but not 5C12 the toxin colocalized with the Golgi body (yellow colored; −5C12). However, the toxin in the presence of 5C12 localized to compartments other than the Golgi body (+5C12). A differential interference contrast image of each fluorescent image is shown on the left side of the figure. This experiment was repeated three times with similar results.

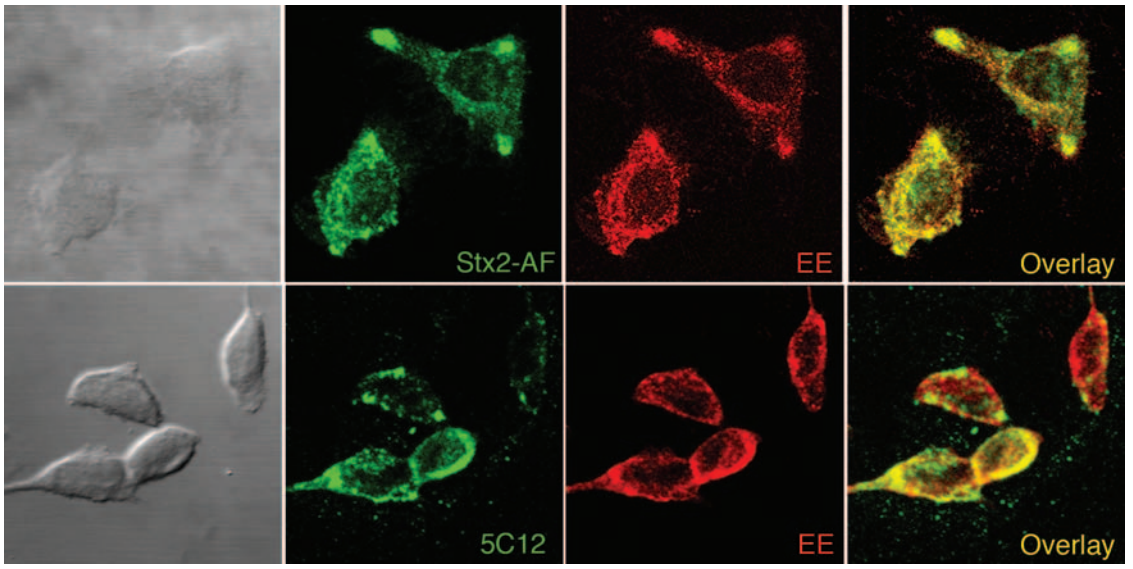

FIG. 8. Stx2/5C12 complex is present in EE. Stx2-AF (upper panel) and 5C12 (lower panel) in Stx2/5C12 complex colocalized with EE after 1 h of incubation at 37°C. A differential interference contrast image of each fluorescent image is shown to the left side of the figure. This experiment was repeated three times with similar results.

bation time to 5 h at 37°C did not affect the localization of the 5C12/toxin complex since the complex was still present in the EE (results not shown). In contrast, in the absence of 5C12, the toxin was mostly found in the ER after 4 h of incubation at 37°C (results not shown).

Stx2 cointernalized with 5C12 localizes to recycling compartments. To investigate whether 5C12 induced the accumulation of the toxin in the recycling compartments, two RE markers, anti-Rme-1 antibody and Tf labeled with AF-568, were utilized. These markers have been used before to localize endocytic recycling compartments, including the PNRC, in HeLa cells (1, 14, 24). To induce accumulation of Tf in recycling compartments, we followed a procedure described previously (26). Briefly, cells that had internalized 5C12/Stx2 complex at 20°C for 1 h and subsequently at 37°C for 4 h were incubated during the last 1 h at 37°C with AF568-labeled Tf. Indeed, 5C12 caused Stx2 to largely accumulate in compartments alongside the nucleus (PNRC), which colocalized with Tf (Fig. 9A, upper row) and Rme-1 (Fig. 9A, lower row).

It is evident that there are few compartments that accumulated Stx2-AF/5C12 (green) but not Tf (red) and vice versa, and therefore they did not colocalize to appear either yellow or orange in the overlay (Fig. 9A, upper panel). However, if the same areas that accumulated Tf also accumulated Stx2-AF/5C12 are compared, they almost always colocalized. We have performed these experiments a few times, and all of the Tf and Stx2-AF/5C12 cannot be colocalized, presumably because the uptake of these two different molecules (Tf, and Stx2-AF/5C12) cannot be completely synchronized. Furthermore, there may be recycling compartments that are shared by both Tf and 5C12/Stx2 complexes and those that are not. This proposition is also supported by the Rme-1 staining profile since the compartments alongside the cell membrane show the accumulation of Stx2-AF/5C12 but they do not stain for Rme-1(Fig. 9A, lower row). It is clear that there are recycling compartments alongside the cell membrane which rapidly release the internalized Stx2-AF/5C12 complex into the extracellular environment (see the results of the studies below).

Stx2 cointernalized with 5C12 is apparently recycled back to the plasma membrane and released into the extracellular environment. Next, we investigated whether Stx2 excretion was occurring because of the fusion of Stx2/5C12 containing RE with the cell membrane. Briefly, Stx2-AF/5C12 complexes on the cell surface were allowed to internalize for 1 h at 20°C. Cells were then washed and cultured at 37°C for 4 h either in fresh medium, or in fresh medium containing the B-subunit-specific HuMAb 5H8 (250 μg/ml) to determine whether presence of extracellular 5H8 would reduce binding of the excreted Stx2-AF/5C12 complexes to the cells and prevent their reinternalization. At 0 h (control for Stx2-AF/5C12 internalization) and 4 h of incubation at 37°C, the cells were fixed and analyzed by confocal microscopy (Fig. 9B) and flow cytometry (Fig. 9C). Confocal microscopy revealed that in the presence of extracellular 5H8, fluorescence due to Stx2-AF almost completely disappeared from alongside the membrane but was present in intracellular compartments, presumably the PNRC (Fig. 9B). Such a decrease in intracellular Stx2-AF due to the presence of extracellular 5H8 was also observed by flow cytometry (Fig. 9C) since the fluorescence peak obtained in the absence of 5H8 (green histogram) shifted toward a region of low fluorescence intensity when 5H8 was present (blue histogram).

Live cell microscopy. To further confirm the influence of 5C12 on retrograde transport of the toxin, we conducted microscopy on living cells (see videos S1 and S2 in the supplemental material).

Video S1 in the supplemental material shows intracellular transport of Stx2-AF in absence of 5C12. Seven cells are in frame, and the images were captured over 60 min. Initially, Stx2-AF spread out uniformly in cellular organelles (presumable EE), and then it gradually accumulated around the nuclei (presumably Golgi as demonstrated in Fig. 7).

Video S2 shows intracellular transport of Stx2-AF in pres-

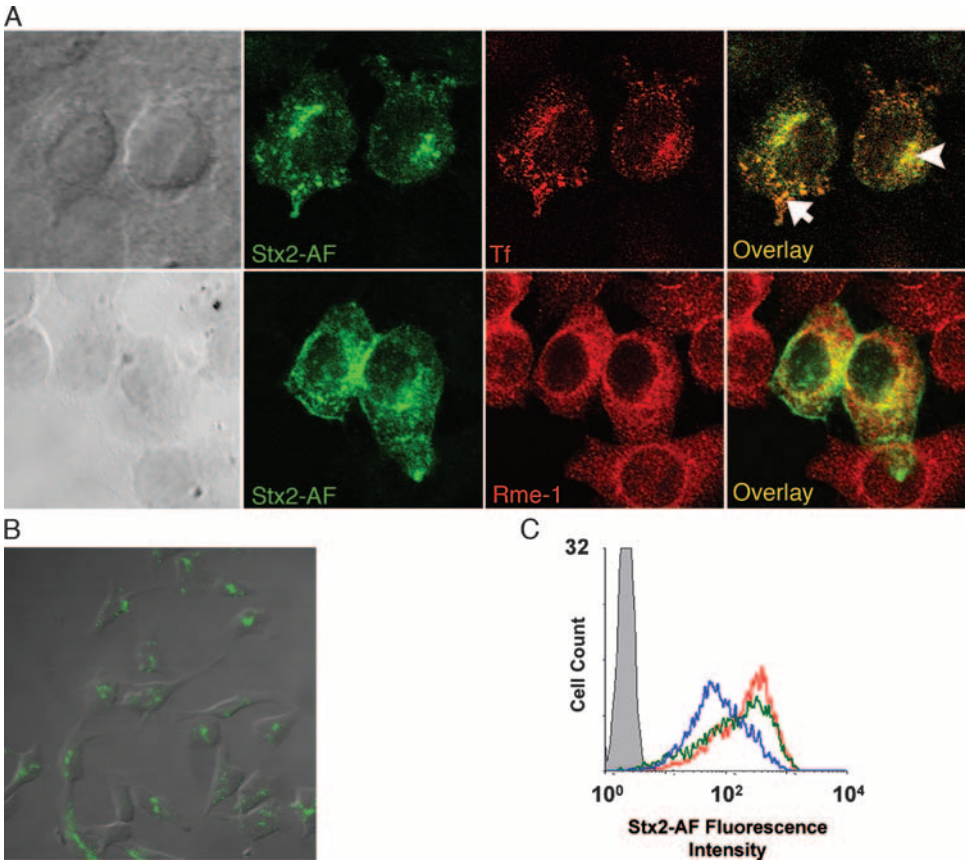

FIG. 9. Stx2/5C12 complex enters endocytic recycling compartments and recycles back to the cell surface for release into the extracellular environment. (A) Stx2-AF in Stx2-AF/5C12 complex colocalized with Tf-AF568-labeled (upper row) and Rme-1-labeled (lower row) recycling compartments. The colocalization was mostly in compartments around the nuclei (perinuclear recycling compartments, PNRC; indicated by an arrowhead in the upper row), and some in other recycling compartments in the cytoplasm (shown by an arrow in the upper row). However, the Stx2-AF/5C12 complex that localized in compartments alongside the membrane did not localize with Rme-1 (lower row). (B) Cells with internalized Stx2/5C12 complexes were incubated at 37°C in medium containing 5H8 (250 μg/ml) for 4 h. Most of the Stx2-AF/5C12 complex, especially around the cell membrane, disappeared from the HeLa cells. The leftover Stx2-AF/5C12 complex seems to be present mostly in compartments around the nucleus (PNRC). (C) The cells from the experiment in panel B were also analyzed by flow cytometry, and a marked decrease in intracellular Stx2 was observed in the presence of extracellular 5H8 (blue histogram) compared to its absence (green histogram). The red histogram shows intracellular Stx2-AF at the 0-h time point in the absence of extracellular 5H8. The gray histogram represents unlabeled cells. The experiments were repeated three times with similar results.

ence of 5C12. Six cells are in the frame. At 60 min, most of the toxin was in a perinuclear compartment of at least two (bottom left) of the six cells, and this compartment should be the PNRC, as seen in Fig. 9A. Whereas the cells at the top left and top center and the upper cell of the two bottom right cells have considerable amount of toxin in the PNRC and also some in the compartments alongside of the cell membrane, the cell at the bottom right has the most toxin in the compartments alongside of the cell membrane. These observations suggest that HeLa cells in a monolayer are not synchronous or identical in recycling the toxin/5C12 complexes and confirm the accumulation of Stx2/5C12 in recycling compartments alongside the cell membrane and nucleus (the PNRC) before excretion into the extracellular environment.

DISCUSSION

In this study, we discovered a unique mechanism of intracellular toxin neutralization by an antibody to the A subunit of Stx2. Although both Stx2 A-subunit- and B-subunit-specific HuMAbs 5C12 and 5H8, respectively, neutralized Stx2, the neutralizing mechanisms were distinct. The HuMAb 5H8 inhibited cytotoxicity by preventing binding of the unbound toxin to the cells but was ineffective against the cell bound toxin. In contrast, the A-subunit-specific 5C12 intercepted the cell-bound toxin and prevented toxin-mediated cell death.

Since Stx2 must be endocytosed and translocated to the cytosol by retrograde transport to exert its cytotoxic effect, a block in any step in this process of toxin transport by 5C12 may lead to inhibition of cytotoxicity. Before investigating this possibility, we determined the route and time course of Stx2 transport through the cellular organelles of HeLa cells because the sequence of retrograde transport has been studied for Stx (8, 12, 17) and Stx1 (29) but not for Stx2. Our results indicate that in HeLa cells Stx2 follows the same direct route of retrograde transport as that followed by Stx B subunit in these cells (8, 17). Stx2 starts accumulating in the EE soon after incubation at 37°C. It accumulates in the Golgi compartment after 1 h of

incubation and in the ER after 4 h of incubation at 37°C; the results are in agreement with the time required for Stx B subunit to accumulate in these organelles in HeLa cells (8, 17). Stx B subunits have been shown to bypass the late endocytic pathway on their way to the Golgi apparatus in HeLa cells (8, 17). We also could not locate Stx2 in LE/lysosomes in HeLa cells (results not shown).

5C12 does not interfere with the endocytosis of Stx2, since Stx2/5C12 complexes were detected in the EE. However, 5C12 blocks further passage of the toxin into other cellular organelles, since even after incubation for 5 h at 37°C, the toxin was mainly located in the EE. The same results were obtained after 8 h of incubation at 37°C (results not shown). We were not able to locate Stx2-AF/5C12 complex in the LE and/or lysosomes since it was not found in LAMP3-positive structures (results not shown). It was clear from these results that 5C12 interfered with retrograde transport of the toxin. Further studies were performed to determine the fate of the internalized Stx2/5C12 complex.

Internalized membrane molecules are either targeted for degradation or recycled back to the plasma membrane (18). In contrast, endocytosed nonmembrane molecules are usually targeted for degradation, but some molecules evade that route completely and enter the RE in order to be released outside the cell (18). These studies suggested to us that the Stx2/5C12 complex may be in the RE. The two types of RE compartments identified thus far include those that recycle molecules rapidly to the plasma membrane and those that recycle molecules slowly (the PNRC) to the plasma membrane (18). It seems that the 5C12-treated Stx2 localized in both the compartments alongside the plasma membrane (presumably rapid RE) and the PNRC (Fig. 9A and Video S2 in the supplemental material). This suggests that the endocytosed complex follows two routes of repeated recycling; one involves rapid transport to and then endocytosis from the plasma membrane, and another involves slow transport from the PNRC to and then endocytosis from the plasma membrane. Videos S1 and S2 in the supplemental material also suggest that toxin/5C12 complex accumulates in endocytic recycling compartments. In some cells, the complex may preferentially recycle either through the PNRC or through the rapid RE, whereas in other cells the recycling seems to be distributed equally among the two compartments.

During fusion of RE with the cell membrane, the Stx2/5C12 complex may come off because the toxin detaches from Gb_3. The toxin could then reattach to any Gb_3 receptor, leading to the endocytosis of the complex again. We investigated this hypothesis by incubating the cells that had endocytosed Stx2-AF/5C12 complex with B subunit-specific 5H8. Since 5H8 blocks the binding of Stx2 with the cells and can bind to Stx2/5C12 complex (enzyme-linked immunosorbent assay results [not shown]), we anticipated 5H8 to block the binding of detached Stx2-AF/5C12 complex with the Gb_3. The presence of 5H8 caused a marked reduction in overall fluorescence (Fig. 9B and 9C), which clearly suggests that Stx2/5C12 complex is transported to the cell membrane, where a new cycle of its internalization is blocked by 5H8. The results also showed that the fluorescence, although present inside the cell (presumably in the PNRC), almost completely disappeared from alongside the plasma membrane, which suggests that rapidly recycled complex had more chances for neutralization by 5H8 than the slowly recycled complex from the PNRC because they were trapped there for a longer time.

Although the mechanism by which 5C12 makes Stx2 quit the retrograde transport and follow the recycling transport needs to be investigated, it is tempting to speculate and also exclude some possibilities. Major histocompatibility complex class I-like Fcγ receptor (FcRn) is known to bind serum IgG and recycle it back to the plasma membrane, rescuing it from lysosomal degradation (15). Since HeLa cells do not express FcRn (34), 5C12/Stx2 recycling cannot be FcRn mediated. It is well understood that TfR accumulates in recycling compartments after endocytosis and recycles back to the plasma membrane (18). However, the intracellular transport of Gb_3 has not been studied. It is possible that Gb_3, like TfR and some other host cell surface molecules, may be destined to be recycled back to the cell surface. Since Stx2 is known to bind to Gb_3 at low affinity (32), it may come off at low endosomal pH to follow the retrograde transport. However, binding of 5C12 may confer conformational changes in the Stx2 molecule, leading to slightly stronger binding of the Stx2 with the Gb_3. We have observed that the binding of Stx2 with its receptor in the presence of 5C12 is somewhat stronger than in its absence (Fig. 2).

The present study is the first to report intracellular neutralization of a toxin by an antibody. Also, the mechanism of antibody neutralization described here has not been reported earlier, although several studies have shown that antibodies can neutralize pathogens intracellularly (3, 4, 6, 19, 20, 33). We speculate that 5C12 will also protect Stx2-bound cells in vivo. This would make 5C12 effective for patients where a toxin dose sufficient to initiate kidney damage has been absorbed systemically from the gut. Our recent study in which 5C12 protected piglets against Stx2-mediated lethal neurological complications wherein 5C12 was administered 48 h after oral infection with STEC supports this hypothesis (30). Furthermore, 5C12 protects 20 to 40% mice when administered after 1 to 2 h of intravenous challenge with a lethal dose of Stx2 (unpublished results). We believe that the 5C12/Stx2 complex will ultimately be removed by the reticuloendothelial system in vivo and will not keep recycling in the cells. Studies are currently being performed to investigate the in vivo sites of Stx2 neutralization by 5C12 and the removal of 5C12/Stx2 complexes from the body.

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